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HEMOPOIETIC AND IMMUNOLOGIC MECHANISMS

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Part A: Development of general and specific immunopotentiators

We carried out work on the regulation of IgE production in humans by lymphokines. The work has been very successful and has been received enthusiastically by the scientific community.

IgE is a very important immunoglobulin since it is the only one involved in the defence against helminthic infections. Furthermore IgE is involved in all allergic reactions. IgE has been recently shown to participate in the defence against viral infections. Our work has focused on the characterization of basic mechanisms which are involved in the regulation of the production of IgE by human B cells. Identification of substances able to up- or -down regulate IgE production may allow the planning of proper clinical trials in the future.

The cytokine interleukin 4 (IL-4) has been shown to induce lipopolysaccharide-activated murine B cells to differentiate into IgE-secreting cells and to stimulate IgE secretion by cultured human peripheral blood lymphoid cells. It is unclear, however, whether this effect of IL-4 on human peripheral blood lymphoid cells is a direct effect on the B cell because IL-4 can stimulate T cells and monocytes as well as B cells and dose not induce purified human B cells to secrete immunoglobulin. To investigate this issue we studied the ability of IL-4 to induce IgE secretion by purified human B cells (93-96% CD20$^+$, <1% CD3$^+$) that were cultured with Epstein-Barr virus (EBV). Although B cells cultured with IL-4 alone did not secrete Ig and B cells cultured with EBV alone secreted IgM, IgG and IgA but <150 pg of IgE per ml, the combination of EBV and IL-4 induced an IgE response that

ranges from 11.4 to 40.3 ng/ml of culture supernatant after 26 days of culture. While IL-4 also enhanced IgM, IgG, and IgA secretion, as well as proliferation by EBV-infected B cells, these effects were less pronounced, occurred earlier during culture, and required a lower concentration of IL-4 than did the stimulation of IgE secretion. Furthermore, interferon gamma at 10 units per ml was found to inhibit IL-4/EBV-induced IgE secretion without inhibiting the other stimulatory effects of IL-4 and interferon-gamma can act directly on polyclonal activated human B cells to respectively stimulate and suppress IgE secretion and IL-4, in addition to its specific effect of IgE secretion, has a general stimulatory effect on the growth and differentiation of EBV-infected human B cells.

To identify the time period during which EBV-infected B cells can be induced by -IL-4 to secrete IgE, we have studied the effects of delayed addition of IL-4, or the termination of IL-4 stimulation by wash out or by neutralization with anti-IL-4 antibodies, on the induction of an IgE responses. To induce a maximal IgE response, IL-4 had to be added to cultures of B cells plus EBV no later than two days after the initiation of culture, and had to remain present through the tenth day of culture. These two time points correspond to the initiation of detectable DNA synthesis (day two to three) and the earliest detectable Ig secretion (day two to eleven) by EBV-stimulated B cells. No IgE response was induced if the period during which EBV-stimulated B cells were cultured with IL-4 was less than four days, or if IL-4 were added later than the tenth day of culture, regardless of how

long the culture was continued beyond that time. In contrast, IL-4 considerably enhanced IgG and IgM secretion and B cell CD23 expression, even if it was added after the tenth day of culture. IFN-gamma strongly inhibited the IgE response of B cells cultured with IL-4 plus EBV if added within six days of the initiation of culture, but had little effect on the generation of IgM of IgG responses made by these cells, regardless of the time of addition. Neither IL-4 nor IFN-gamma affected ongoing IgE secretion by an established, IgE-secreting, EBV-transformed cell line. These observations suggest that: 1) IL-4 first becomes able to induce EBV-activated B cells to secrete IgE as these cells begin to synthesize DNA, must stimulate B cells for at least four days to induce IgE secretion, and loses it ability to induce IgE secretion as these cells differentiate into Ig-secreting cells; 2) the ability of IFN-gamma to suppress an IgE responses is limited to this same time period; and 3) IL-4 enhancement of CD23 expression and IgM and IgG secretion are independent of IL-4 induction of an IgE response.

Publications

1.   Thyphronitis G., Tsokos G.C., June C.H., Levine A.D., Finkelman F.D. IgE secretion by Epstein-Barr virus-infected purified human B lymphocytes is stimulated by interleukin-4 and suppressed by interferon-gamma. Proceedings of the National Academy of Sciences, USA, Vol. 86, pp 5580-5584, 1989. (Appendix I)

2.   Thyphronitis G., Banchereau J., Heusser C., Tsokos G.C.,

Levine A.D., Finkelman F.F. Kinetics of interleukin-4 induction and interferon-gamma inhibition of IgE secretion by Epstein-Barr virus-infected human peripheral blood B cells. <u>Cellular Immunology</u>, (Appendix II)

B. Hemopoietic and Survival Studies in Immunosuppressed Mice

As described in the original proposal, studies to evaluate selected USAMRIID AVS agents for hemopoietic effects and for the ability to nonspecifically enhance resistance to opportunistic infections in immunosuppressed hosts were evaluated by Dr. M. L. Patchen at the Armed Forces Radiobiology Research Institute (AFRRI). The ability of various agents to stimulate hemopoiesis at the level of the pluripotent hemopoietic stem cell was evaluated using the endogenous spleen colony-forming unit assay (E-CFU assay). In this assay, the hemopoietic stem cell content of an animal is reduced by exposure to a sublethal dose of irradiation. Experimental drugs are administered either before (prophylactic drug treatment) or after (therapeutic drug treatment) the hemopoietic injury and evaluated for the ability to accelerate hemopoietic regeneration. Increases in E-CFU numbers indicate a drug's ability to function as a hemopoietic stimulant. The second parameter assayed in studies performed at AFRRI was the ability of the same selected AVS agents to prophylactically or therapeutically enhance survival in severely irradiated mice. Since following radiation doses such as those used in these experiments, death results from opportunistic pathogens, these studies provide an indication of the ability of an agent to enhance nonspecific resistance in an immunocompromised host.

During this contract 14 USAMRIID AVS agents were evaluated for these parameters. Results of these studies were summarized in the following two table and four figures. The most effective

agents, taking both prophylactic and therapeutic efficacy into account were Xerosin, AVS 2776, and AVS 2777.

Publications: None

BRM DOSAGES

| AVS #	ug/kg			mg/kg											
	1.25	2.5	5	2.5	5	10	12.5	20	25	40	50	100	200	300	400
?	▓						▓		▓		▓				
00001	▓										▓	▓	▓		
1758												▓	▓		▓
1968						▓		▓		▓					
2149				▓	▓										
2776													▓	▓	▓
2777													▓	▓	▓
2778													▓	▓	▓
3926							▓		▓		▓				
3927							▓		▓		▓				
3933															
3934											▓	▓	▓	▓	
3960	▓	▓	▓						▓		▓		▓		
4054															

EP136T

Comparative E-CFU and Survival Enhancing Effects of Tested BRM's (optimal drug doses)

| BRM	mg/kg	Prophylactic Treatment		Therapeutic Treatment	
		E-CFU	% Survival	E-CFU	% Survival
Saline	-	4.0 ± 0.9	5	4.0 + 0.9	40
AVS ?	50.0	30.3 ± 2.2	70	>40	85
AVS 00001	50.0	6.3 ± 2.8	30	8.0 ± 1.5	35
AVS 1758	400.0	>40	40	>40	15
AVS 1968	40.0	4.7 ± 1.6	15	5.7 ± 1.5	45
AVS 2149	2.5	3.8 ± 1.7	25	5.7 ± 1.2	30
AVS 2776	300.0	19.6 ± 2.5	80	24.7 ± 2.8	85
AVS 2777	300.0	32.3 ± 3.2	80	30.5 ± 1.3	90
AVS 2778	300.0	25.3 ± 4.9	90	10.5 ± 1.3	0
AVS 3926	25.0	... ± 2.3	20	17.4 ± 2.3	70
AVS 3927	25.0	... ± 1.4	20	11.6 ± 1.7	70
AVS 3933	100.0	... ± 1.5	50	15.2 ± 2.3	45
AVS 3934	300.0	... ± 1.3	30	10.9 ± 1.3	50
AVS 3960	50.0	14. ± 3.3	0	15.5 ± 4.2	20
AVS 4054	2.5(ug/kg)	... ± 1.8	0	15.2 ± 1.3	60

EP135T

# Comparative Effects of Tested BRM's on Survival in C3H/HeN Mice Following 8.5 Gy (Prophylactic Administration)
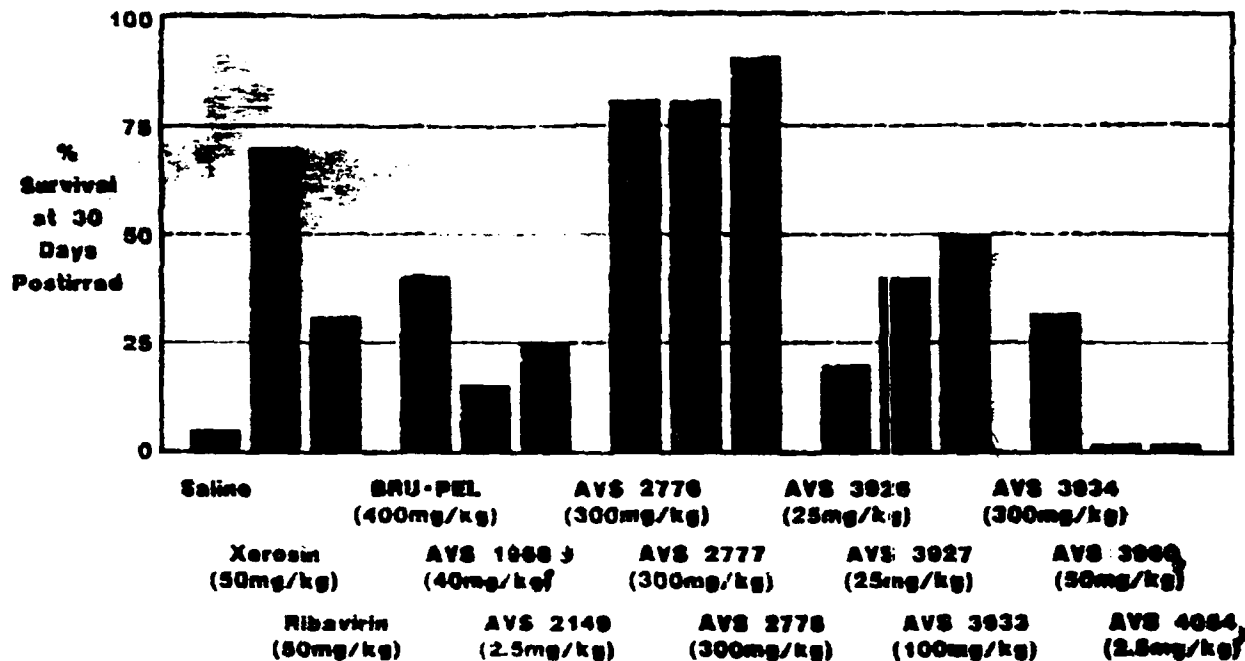
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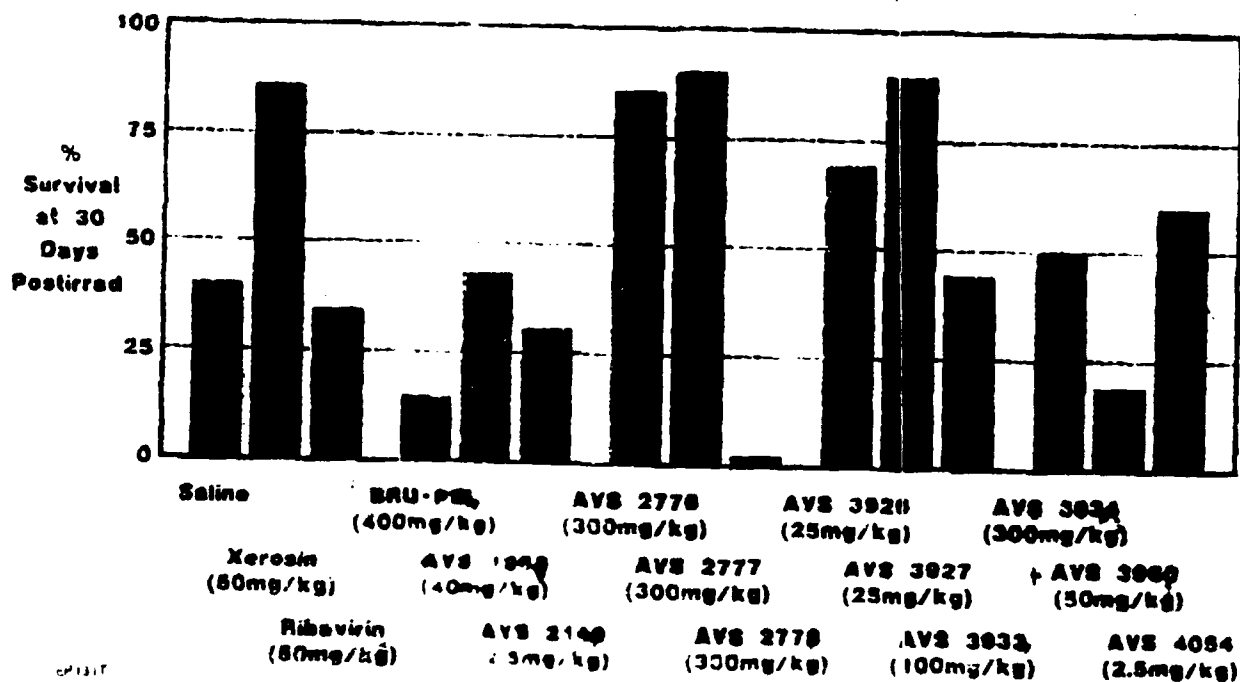
Comparative Effects of Tested BRM's on Hemopoietic Stem Cell Regeneration in C3H/HeN Mice Following 6.5 Gy (Prophylactic Administration)
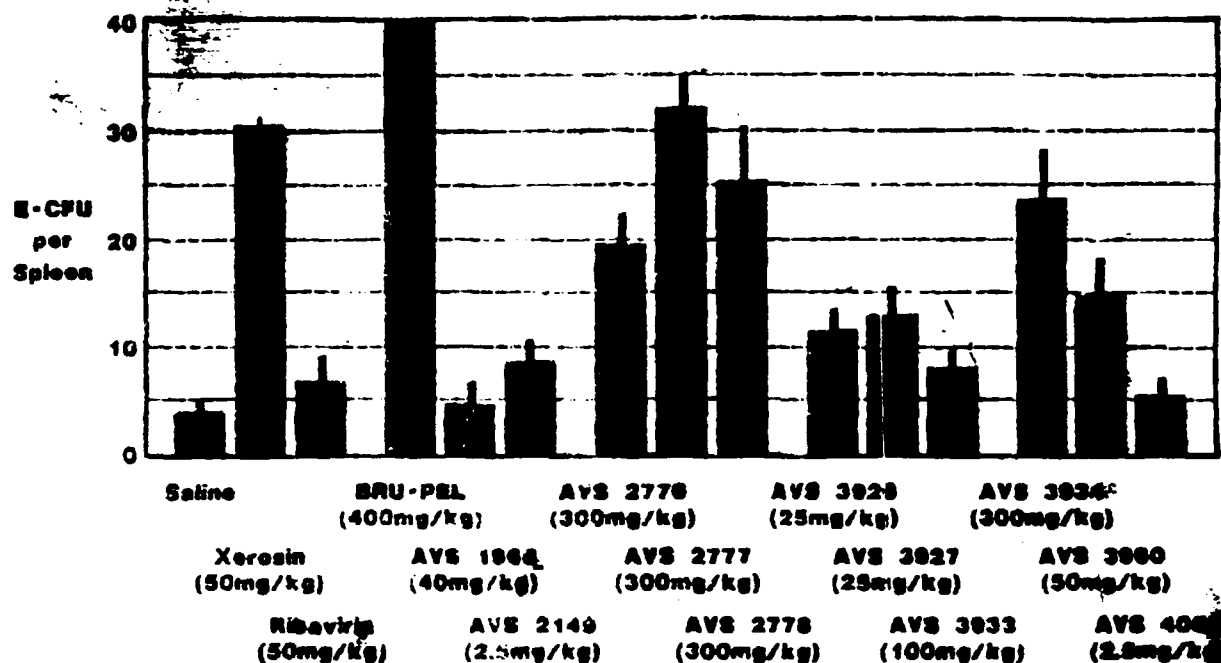
Comparative Effects of Tested BRM's on Hemopoietic Stem Cell Regeneration in C3H/HeN Mice Following 6.5 Gy (Therapeutic Administration)
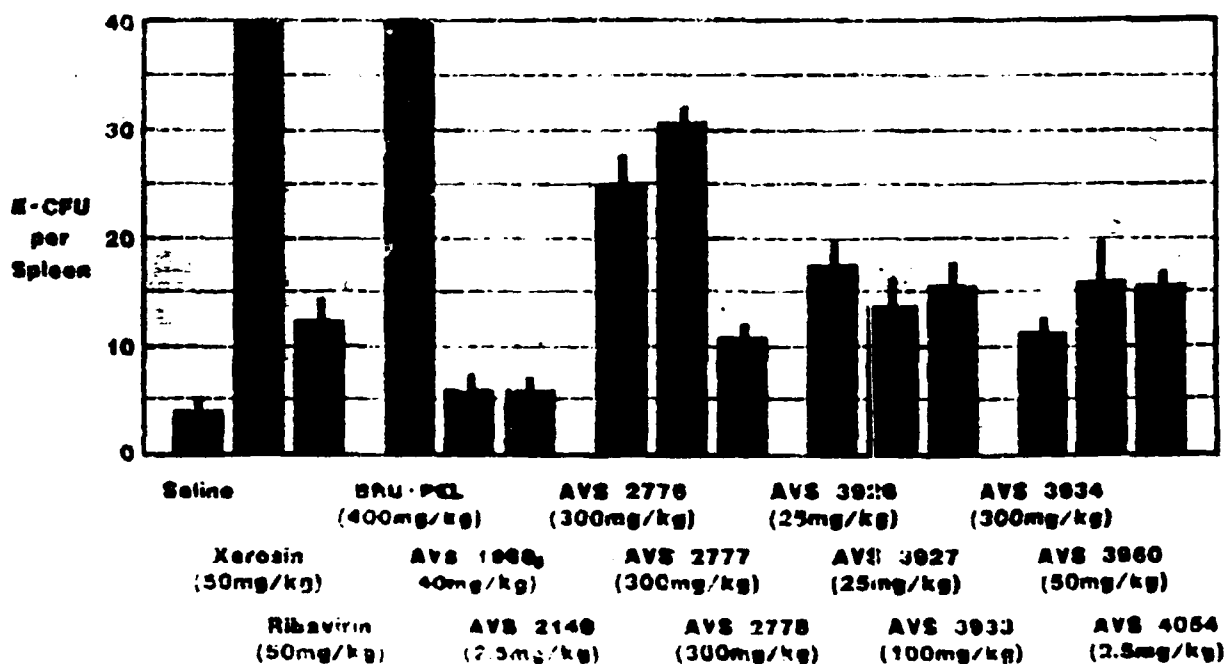
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IgE secretion by Epstein–Barr virus-infected purified human B lymphocytes is stimulated by interleukin 4 and suppressed by interferon γ

(allergy / polyclonal B-cell activation / immunoglobulin isotypes / class switching)

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ABSTRACT   The cytokine interleukin 4 (IL-4) has been shown to induce lipopolysaccharide-activated murine B cells to differentiate into IgE-secreting cells and to stimulate IgE secretion by cultured human peripheral blood lymphoid cells. It is unclear, however, whether this effect of IL-4 on human peripheral blood lymphoid cells is a direct effect on the B cell because IL-4 can stimulate T cells and monocytes as well as B cells and does not induce purified human B cells to secrete immunoglobulin. To investigate this issue we studied the ability of IL-4 to induce IgE secretion by purified human B cells (93–96% CD20+, <1% CD3+) that were cultured with Epstein–Barr virus (EBV). Although B cells cultured with IL-4 alone did not secrete Ig and B cells cultured with EBV alone secreted IgM, IgG, and IgA but <150 pg of IgE per ml, the combination of EBV and IL-4 induced an IgE response that ranged from 11.4 to 40.3 ng/ml of culture supernatant after 26 days of culture. While IL-4 also enhanced IgM, IgG, and IgA secretion, as well as proliferation by EBV-infected B cells, these effects were less pronounced, occurred earlier during culture, and required a lower concentration of IL-4 than did the stimulation of IgE secretion. Furthermore, interferon γ at 10 units per ml was found to inhibit IL-4/EBV-induced IgE secretion without inhibiting the other stimulatory effects of IL-4. We conclude that (i) IL-4 and interferon γ can act directly on polyclonally activated human B cells to respectively stimulate and suppress IgE secretion and (ii) IL-4, in addition to its specific effect on IgE secretion, has a general stimulatory effect on the growth and differentiation of EBV-infected human B cells.

Because IgE plays a critical role in the stimulation of mast cell degranulation and the generation of allergic symptoms that result from this process (1–3), recent evidence that two cytokines regulate IgE secretion may have clinical importance. Studies in the mouse have demonstrated that (i) interleukin 4 (IL-4) can induce purified, lipopolysaccharide-activated B lymphocytes to secrete IgE in the absence of other T cell stimuli (4); (ii) IL-4 is required for T-cell-dependent induction of IgE secretion both in vitro and in vivo (5–7); (iii) IL-4 induces transcription of a germ-line ε chain transcript (8); and (iv) interferon γ (IFN-γ) specifically inhibits the differentiation of B lymphocytes into IgE-secreting cells (9–11). In vitro studies with human lymphocytes support the possibility that these two cytokines also regulate human IgE secretion: (i) Culture of human peripheral blood cells with IL-4 induces an IgE response, which can be inhibited by the addition of IFN-γ to the culture system

(12, 13); (ii) culture of purified human B cells with cloned helper T cells induces an IgE response that is directly proportional to the quantity of IL-4 produced by the T cells and inversely proportional to the quantity of IFN-γ produced by these cells (14). However, because all these studies with human cells required the presence of T lymphocytes and monocytes to induce an IgE response and because both IL-4 and IFN-γ can affect the growth of T as well as B lymphocytes (15–18), these studies do not distinguish whether IL-4 and IFN-γ affect IgE secretion directly at the B-cell level or indirectly, by inducing T cells to produce additional cytokines that can stimulate or suppress IgE secretion. Studies in which anti-Ig antibodies, protein A-bearing *Staphylococcus aureus*, or bacterial endotoxin were used to stimulate human B lymphocytes showed no clear direct stimulatory role for IL-4 in inducing an IgE response (19); indeed, interleukin 2 (IL-2), in the absence of IL-4, has been reported to induce purified B cells cultured with *S. aureus* to secrete IgE, and IL-4 has been found to inhibit *S. aureus* plus IL-2-induced secretion of all Ig isotypes, including IgE (20, 21).

To investigate whether IL-4 and IFN-γ can act directly on human B lymphocytes to regulate IgE secretion we have studied a system in which the infection of highly purified normal human peripheral blood B lymphocytes with Epstein–Barr virus (EBV) induces these cells to proliferate and to secrete IgM, IgG, and IgA, but no detectable IgE. Although addition of IL-4 to the culture system enhances cell growth and production of all Ig isotypes, we find that IgE production is enhanced much more than that of the other isotypes. Addition of IFN-γ along with IL-4 to the EBV-stimulated cultures inhibits IgE production without affecting production of the other Ig isotypes.

MATERIALS AND METHODS

B-Cell Purification. Peripheral blood mononuclear cells were obtained by leukopheresis of five healthy normal donors and depleted of polymorphonuclear leukocytes and erythrocytes by centrifugation over a layer of Ficoll/Hypaque. Lymphocytes were partially depleted of macrophages by fractionation in Sepracell-MN according to the manufacturer's instructions (Sepratech, Oklahoma City, OK), then incubated for 45 min with a mixture of mouse monoclonal antibodies 35.1, 64.1, G17.2.8, G10-1, G3-7, FC2.2, and 60.1 (gift of J. A. Ledbetter, Oncogene, Seattle, WA), which are specific for surface markers CD2, CD3, CD4, CD8 (present on T cells), CD7, CD16, and CD11 (present on macrophages

Abbreviations: IL, interleukin; EBV, Epstein–Barr virus; IFN-γ, interferon γ; cIg, intracytoplasmic immunoglobulin; FITC, fluorescein isothiocyanate.
‡To whom reprint requests should be sent at * address.

and/or natural killer cells), respectively (22-25). The cells were then washed and incubated for 1 hr a. 4°C with iron-containing beads conjugated with goat anti-mouse IgG antibody (Dynal, Great Neck, NY) at a ratio of four beads per cell. Cells bound to beads and free beads were removed magnetically from the cell suspension, after which the remaining cells were washed twice before being placed in culture. Immunofluorescence staining, coupled with fluorescence-activated cell sorter analysis, demonstrated that 93–96% of the resulting cell population bore the B-cell-specific surface marker B1 (Coulter), whereas cells that were stained by fluorescein-labeled antibodies specific for T-cell, macrophage, or natural killer-specific markers (Leu 4, Leu M3, Leu 7, and Leu 11; Becton Dickinson) were not detectable (<1%). The absence of cells stained by these fluorescein-labeled antibodies cannot be accounted for by blocking of antigenic determinants with unlabeled monoclonal antibodies because no cells were stained detectably by a fluorescein-labeled anti-mouse Ig antibody.

Analysis of Ig Secretion. Ig levels in supernatants of cultured cells were analyzed by ELISA in 96-well flat-bottomed polystyrene microtiter plates (Dynatech). To assay IgE levels, wells were coated overnight with goat anti-human IgE antibody at 10 µg/ml (ε chain-specific; Cappel Laboratories) in 0.1 M NaHCO₃ buffer, pH 9.6. Wells were washed, blocked with phosphate-buffered saline/0.1% Tween 20/1% bovine serum albumin, and filled with 100 µl of serial 4-fold dilutions of culture supernatants. After a 2-hr incubation step wells were sequentially filled with 100-µl aliquots of a 1:500 dilution of a mouse monoclonal antibody to human IgE (Hybritech), a 1:20,000 dilution of a biotin conjugate of goat anti-mouse IgG antibody (Jackson ImmunoResearch), a 1:2000 dilution of a peroxidase-streptavidin conjugate (BRL), and substrate (3,3',5,5'-tetramethylbenzidine plus H₂O₂; Kirkegaard and Perry, Gaithersburg, MD) according to the manufacturer's instructions. After 10 min the reaction was stopped by addition of 1 M H₃PO₄, and wells were analyzed for absorbance at 450 nm in an ELISA reader (Dynatech). An IgE myeloma p... ... ... (5 ng/ml; gift of R. Wistar, ...val Medi... ...ter ... ...ter) generated an A_{450} of twice the back... ...und level, whereas IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, or IgM myeloma proteins each at up to 50 µg/ml (also gifts of R. Wistar) failed to generate a detectable reaction in this assay. To quantitate IgM, IgG, or IgA in culture supernatants, wells were first coated with goat anti-human Ig antibody at 1 µg/ml and then incubated sequentially with culture supernatant dilutions, peroxidase conjugates of goat antibody to IgM (diluted 1:2000), IgG (1:5000), or IgA (1:2000), respectively (Cappel Laboratories), followed by substrate. Purified human IgA, IgG, and IgM standards were purchased from Cappel Laboratories.

Cytokines. Recombinant human IL-4 was synthesized by transfected C127 mouse mammary tumor cells as a 17.5-kDa glycoprotein (A. R. Seetharam, T. G. Warren, and A.D.L., unpublished work). The protein was purified from serum-free conditioned medium by sequential chromatography on a Pharmacia Fast S column and a Vidac C₄ column. Homogeneity of the purified lymphokine was demonstrated by amino-terminal sequence analysis, PAGE, and elution as a sharp symmetrical peak on a C₄-microbore reverse-phase column (Applied Biosystems). Purified IL-4 at 250 pg/ml had one unit of activity, as defined as that concentration required to stimulate half-maximal expression of membrane CD23 by the human Burkitt lymphoma Jivove cell line. Recombinant interleukin 1 was a gift of Hoffman–La Roche; IL-2 was a gift of Cetus; and interleukin 3, IFN-γ, and interleukin 6 were gifts of the Genetics Institute (Cambridge, MA).

Culture System. Purified B lymphocytes (10⁵) were cultured in 48-well culture plates (Costar) in 1 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO), 2 mM

L-glutamine, gentamicin at 0.5 mg/ml, and 2 × 10⁻⁵ M 2-mercaptoethanol plus varying doses of recombinant cytokines at 37°C in an atmosphere that contained 5% CO₂. Every 4–5 days 50% of the culture medium was replaced with an equal volume that contained the same cytokine concentration. Culture supernatants of the EBV-producing B95.8 marmoset cell line, which contained 3 × 10⁵ infectious units of EBV were added to cells at the start of culture.

Identification of Cells with Intracytoplasmic Ig (cIg). To identify cells that were synthesizing large quantities of Ig, cultured cells were first centrifuged over a layer of Ficoll/Hypaque to remove dead cells, then mixed with formalin-fixed chicken erythrocytes at a 100:1 ratio and centrifuged onto a glass microscope slide with a cytocentrifuge (Shandon Southern Instruments, Sewickley, PA). Cells were air-dried, fixed for 1 hr in methanol at 4°C, dried, and stained for 30 min with fluorescein-labeled mouse monoclonal antibodies to IgM or IgE (Becton Dickinson), washed, mounted in glycerol, and examined with a Leitz Ortholux phase/fluorescence microscope. The percentage of cIgM⁺ cells was determined by counting lymphoid cells under both phase and UV light; the percentage of cIgE⁺ cells was determined by counting under UV light the number of lymphoid cells that displayed green cytoplasmic fluorescence in fields that contained a total of 100 chicken erythrocytes (and, therefore, 10,000 lymphocytes) (7). Chicken erythrocytes were identified by their oval shape and orange nuclear fluorescence.

RESULTS

IL-4 Increases Ig Secretion by EBV-Infected B Lymphocytes. Supernatants of purified B lymphocytes from four or five normal individuals were cultured with EBV for 26 days in the absence of exogenous cytokines; these cultures contained easily detectable quantities of IgM, IgA, and IgG but no detectable IgE (<150 pg/ml) (Fig. 1). Addition of 2 × 10³ units of IL-4 to cultures increased supernatant concentrations of IgM, IgG, and IgA by an average of 11-, 10-, and
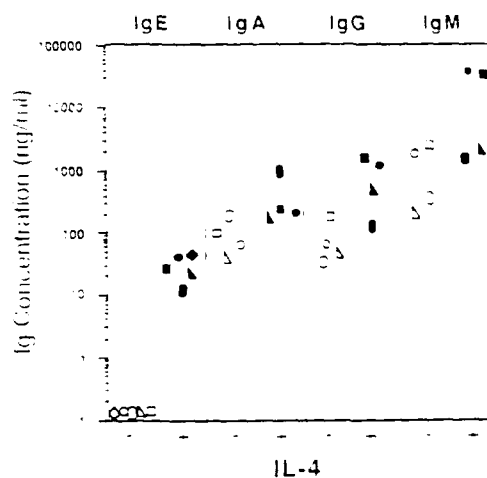

FIG. 1. Effect of IL-4 on EBV-induced Ig secretion. Purified human peripheral blood B cells were cultured for 26 days at 10⁵ cells in 1 ml of culture medium with 3 × 10⁵ infectious units of EBV in the presence or absence of 2 × 10³ units of purified recombinant IL-4. Half of the culture medium was replaced every 4–5 days with fresh medium that contained an equal concentration of IL-4. Day-26 culture supernatants were tested for Ig concentrations by isotype-specific ELISAs. Each symbol represents an individual experiment, and each experiment was performed with cells from a different donor. Open symbols indicate results of cultures that lacked IL-4; closed symbols indicate results of cultures that contained IL-4.

4-fold, respectively. The effect of this cytokine on IgE secretion was much more marked: in the presence of IL-4 culture supernatants had average IgE concentrations of 11.4–40.3 ng/ml, an increase, on average, of >180-fold over baseline (Fig. 1). Thus, while IL-4 increases production of all Ig isotypes by EBV-infected B lymphocytes, the increase in IgE production is at least 10-fold greater than that of other Ig isotypes. In contrast, the addition of recombinant IL-1, IL-2, IL-3, or IL-6 to purified B cells cultured with EBV failed to induce detectable IgE secretion, and B cells cultured with 2–2000 units of IL-4 in the absence of EBV also failed to secrete detectable IgE (data not shown).

IL-4 Increases the Percentage of EBV-Infected B Cells That Synthesize IgE. To determine whether IL-4 increased the percentage of cells that synthesize large quantities of IgE, in addition to the total quantity of IgE secreted, purified B cells cultured for 26 days with EBV in the presence or absence of IL-4 at 2 × 10³ units/ml were fixed on glass slides, stained with fluorescein isothiocyanate (FITC)-labeled anti-IgM or anti-IgE antibodies, and examined by fluorescence microscopy for the percentage of cells with bright intracytoplasmic fluorescence. IL-4 increased the percentage of cIgM⁻ cells from 17 to 41 and increased the percentage of cIgE⁻ cells from <0.010 to 0.267. Thus, the large increase in IgE secretion is accompanied by a sizable increase in the percentage of cells that synthesize large quantities of IgE and most likely secrete this isotype.

Effects of IL-4 Concentration on Increases in Cell Number and Ig Secretion. Although IL-4 caused increases in cell

number and in the concentration of all Ig isotypes tested in culture supernatants, these effects were dissociated to some extent by differences in the dose–response curves (Fig. 2). IL-4 at 2 units per ml stimulated a maximal increase in cell number as well as substantial increases in IgG and IgM concentrations; however, this dose failed to induce detectable IgE secretion. IgE secretion first became detectable when IL-4 was added to cultures at a dose of 6.6 units per ml. Increasing the IL-4 concentration to 20 units per ml stimulated a considerable further increase in IgE secretion, but increases in IL-4 concentration beyond this level had little effect on secretion of IgE. No decreases in secretion of any of the measured Ig isotypes were seen at IL-4 doses as high as 2000 units per ml, although the number of live cells recovered from cultures declined when IL-4 concentrations of 66 units per ml or higher were used. These observations suggest that the mechanisms through which IL-4 induces increases in cell number, the secretion of IgE, and the secretion of other Ig isotypes may differ; these observations further indicate that increased IgE secretion cannot simply be a consequence of an IL-4-induced increase in lymphocyte proliferation or survival.

IgE Is Produced Late by B Cells Cultured with EBV Plus IL-4. By the 10th day of culture of B cells with EBV plus IL-4, the number of cells per culture increased by a factor of 4–5, and considerable quantities of IgM and IgG accumulated in the culture supernatant (Fig. 3). In contrast, IgE was either not detectable (<150 pg/ml) or just barely measurable in culture supernatants at this time (280 pg/ml in the experiment shown in Fig. 3). In addition, the percentage increase in culture supernatant IgE concentration between days 10 and 14 was considerably greater than the percentage increases in the culture supernatant concentrations of IgM or IgG during this time period. Thus, induction of IgE secretion is regulated differently from induction of IgG and IgM secretion and may
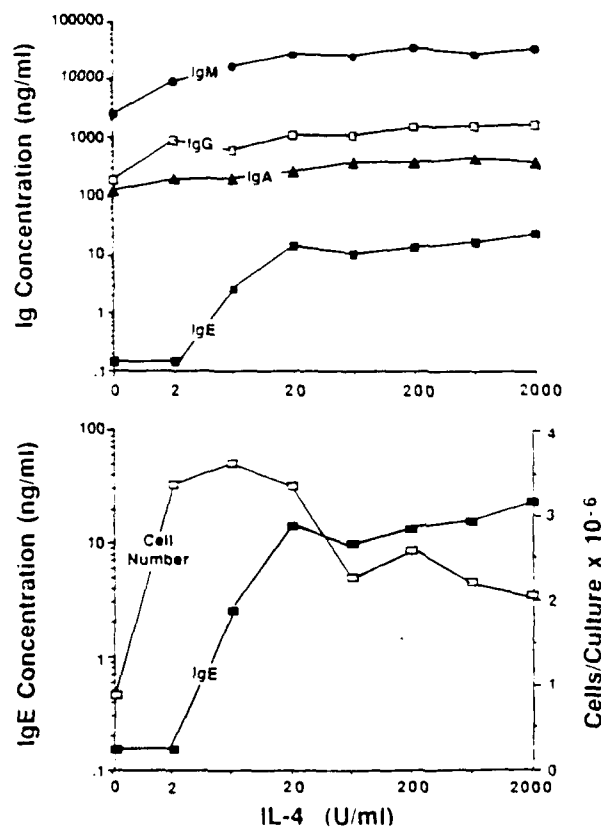

FIG. 2. Effect of IL-4 concentration on proliferation and Ig secretion by EBV-infected human B cells. Purified human peripheral blood B cells were cultured as described in the legend to Fig. 1 with EBV plus various concentrations of IL-4. Half of the culture medium was replaced every 4–5 days with fresh medium that contained an equal concentration of IL-4. Day-26 culture supernatant Ig concentrations were determined by ELISA; live cell counts were determined by trypan blue exclusion. Upper and lower panels show data from the same experiment. U, units.
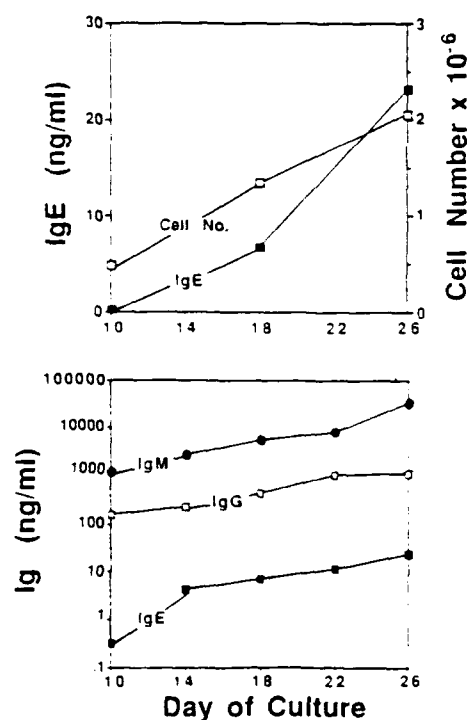

Day of Culture

FIG. 3. Kinetics of proliferation and Ig secretion by EBV/IL-4-stimulated B cells. Purified human peripheral blood B cells were cultured with EBV plus IL-4 at 2 × 10³ units per ml as described in the legend to Fig. 1. Ig concentrations in culture supernatants were determined by ELISA; cell counts were performed in the presence of trypan blue to determine numbers of live cells. Upper and lower panels show data from the same experiment.

require more prolonged stimulation with EBV plus IL-4 than does induction of B-cell growth or IgM or IgG secretion.

T Cells Do Not Increase in Number During the Culture of Purified B Cells with EBV and IL-4. Because T cells have been required for the generation of an IgE response by cultured human lymphoid cells in other systems and because the generation of an IgE response by purified B cells cultured with EBV plus IL-4 takes >10 days, it was necessary to determine whether T lymphocytes, although undetectable at the initiation of culture, increase in number during this time period. To investigate this possibility we stained cells after 8 and 16 days of culture with FITC-labeled antibodies specific for T cells (anti-CD3), B cells (anti-CD20), or with a FITC-labeled control antibody and then analyzed these cells for fluorescence intensity with a fluorescein-activated cell sorter. Less than 1% of cells from either group were stained significantly by anti-CD3, whereas a large percentage of unfractionated peripheral blood mononuclear cells that had been cultured for 8 days with IL-4 plus EBV were stained brightly by this reagent (Fig. 4). Furthermore, virtually all cells cultured with EBV and IL-4 continued to bear the B-cell marker CD20 after 8 or 16 days of culture. Thus, induction of IgE secretion by the combination of EBV and IL-4 cannot be explained by T-lymphocyte outgrowth during culture.

IFN-γ Specifically Inhibits IgE Production by B Cells Cultured with EBV Plus IL-4. Because IFN-γ inhibits IL-4 effects in many murine and human systems (9, 10, 12, 26), we studied the effect of this cytokine on the induction of proliferative and Ig-secretory responses by B cells cultured with EBV plus IL-4. At doses >100 units per ml, IFN-γ blocked all B-cell proliferative and Ig-secretory responses to EBV plus IL-4 or to EBV alone (data not shown), presumably because the antiviral effects of IFN-γ interfered with EBV infection of B lymphocytes. However, at 10 units per ml, IFN-γ inhibited IgE secretion by 95% without affecting secretion of IgG or IgM (Fig. 5) or B-cell proliferation (data not shown). Thus, induction of an IgE response is more sensitive to the inhibitory effects of IFN-γ in this system than are some other events stimulated by IL-4. Although IFN-γ probably inhibits IgE production in this system by specifically blocking IL-4 induction of an IgE response, we cannot rule out the possibility that IFN-γ selectively prevents EBV infection of those B cells that can act as precursors of IgE-secreting cells.

FIG. 5. IFN-γ specifically suppresses IgE secretion by EBV/IL-4-stimulated human peripheral blood B cells. Purified human peripheral blood B cells were cultured for 26 days as described in the legend to Fig. 1 with EBV alone, EBV plus IL-4 at 2 × 10³ units per ml or EBV plus IL-4 at 2 × 10³ units per ml and IFN-γ at 10 units per ml. Half of the culture medium was replaced every 4–5 days with fresh medium that contained an equal concentration of cytokines. Culture supernatant Ig concentrations were determined by ELISA. Horizontal lines indicate Ig concentrations of supernatants of cells cultured with EBV alone, open bars indicate Ig concentrations of supernatants of cells cultured with EBV plus IL-4, and shaded bars indicate Ig concentrations of supernatants of cells cultured with EBV plus IL-4 and IFN-γ.

DISCUSSION

Our studies show that highly purified human peripheral blood B lymphocytes can be stimulated by EBV plus the cytokine IL-4 to secrete IgE. EBV, in the absence of IL-4, induced the secretion of IgM, IgG, and IgA, but not IgE, whereas IL-4, in the absence of EBV, failed to stimulate any Ig secretion. These observations are analogous to the results of previous studies in the mouse, which demonstrated that (i) the combination of IL-4 and lipopolysaccharide induces purified B cells to secrete IgE as well as IgM and IgG; (ii) lipopolysaccharide in the absence of IL-4 stimulates secretion of IgM and IgG, but not IgE; and (iii) IL-4, by itself, does not stimulate Ig secretion (4, 27). We do not yet know whether IL-4 modifies the distribution of IgG subclasses secreted by human EBV-infected B cells, as it does for murine lipopolysaccharide-activated B cells (27–29); nor do we have an explanation for the long time required for the combination of EBV and IL-4 to induce a detectable IgE response by human peripheral blood B cells. This delay in the secretion of IgE may explain an earlier report that human B cells do not secrete IgE in response to EBV plus IL-4 (30) because the culture period in that study was only 10 days and we have frequently failed to detect IgE secretion before 14 days of culture. Another cause for failure to detect IgE secretion by B cells cultured with EBV plus IL-4 is that impure IL-4 preparations may contain an inhibitor of IgE secretion; partially purified preparations of recombinant IL-4 used in our initial studies required much higher concentrations of IL-4 to induce IgE secretion than did the highly purified recombinant IL-4 preparations used for the studies described here.

The results of our study complement previous reports that human peripheral blood cells are stimulated by IL-4 to secrete IgE, provided that T cells are present, and that human T-cell clones that secrete IL-4 can induce purified B cells to secrete
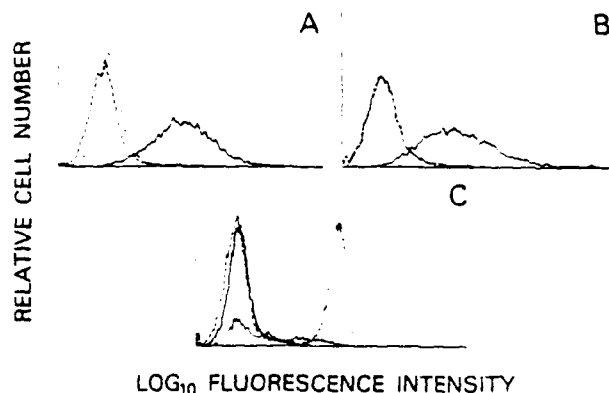
FIG. 4. No increase in T-cell number occurs during culture of purified human peripheral blood B cells with EBV plus IL-4. Purified human peripheral blood B cells were cultured as described in the legend to Fig. 1 for 8 days (A) or 16 days (B) stained with FITC-B1 (—), FITC-Leu-4 (anti-CD3) (···), or as a negative control, FITC-normal mouse IgG (- - -), and analyzed for fluorescence intensity by flow microfluorimetry. The flow microfluorogram of similarly stained and analyzed unfractionated peripheral blood lymphocytes that were cultured for 8 days with EBV plus IL-4 is shown in C.
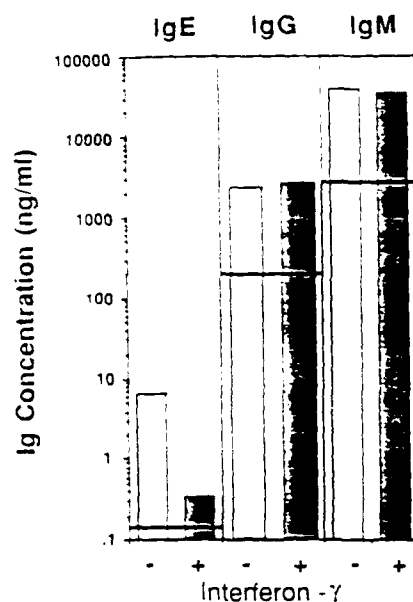
IgE (12-15). Because IL-4 stimulates human T cells as well as B cells (15), these studies left open the possibility that the IL-4 stimulatory effect on IgE secretion was entirely indirect, in that IL-4 stimulated T cells to secrete a second lymphokine that induced B cells to secrete IgE. The T-cell requirements for the induction of a human IgE response were also unresolved by a recent report in which EBV-infected B cells, cultured at low density with activated, irradiated "filler" T cells, were induced to secrete IgE, because the capacity of the filler cells to provide either contact-mediated or cytokine help was not defined (31). In contrast, the absence of any detectable T-cell population at the initiation of our cultures, as well as at 8 and 16 days later in our system, eliminates the possibility that any form of T-cell help other than IL-4 is required to induce EBV-infected B cells to secrete IgE. In addition, the lack of T cells in our system allowed the demonstration that the inhibitory effect of IFN-γ in human, as in mouse (9, 10), can operate directly at the B-cell level. Our system should similarly be useful for investigations of whether IgE binding factors, such as soluble CD23 (32), work directly at the B-cell level to stimulate IgE secretion (13, 33) and may allow the cloning of IgE-secreting, EBV-transformed cells (34-36). Such cells would be useful for determining whether IL-4 and IFN-γ continue to regulate IgE secretion by a cell at this stage of differentiation as well as for investigating whether induction of IgE secretion by EBV-infected cells is accompanied by deletion of upstream Ig heavy chain constant region genes. Investigation of the status of Ig heavy chain constant region genes upstream to the gene for the constant region of the ε chain in B cells induced by EBV plus IL-4 to secrete IgE has become particularly important in view of the recent report that no deletion of these genes occurs in B cells that secrete IgE when infected with EBV and cultured with irradiated, activated T cells (31).

Stimulation of IgE secretion was not the only effect of IL-4 on the growth and differentiation of EBV-infected B cells in our study; this cytokine also substantially increased B-cell proliferation and the secretion of IgM, IgG, and IgA. These additional effects, however, may operate through a different mechanism than does the stimulation of IgE secretion because (*i*) these effects occur earlier than IgE secretion, (*ii*) as in the mouse these effects can be induced by lower IL-4 concentrations than are required to induce IgE secretion, and (*iii*) these effects are not inhibited by a concentration of IFN-γ that effectively blocks IgE secretion. These considerations are relevant to the use of agents that inhibit IL-4 production or the effects of IL-4 on the B cell as possible therapies for IgE-mediated disorders because they show that it is sometimes possible to block IL-4-mediated IgE secretion without inhibiting other effects of IL-4. Also of interest in this regard is the observation in murine *in vivo* studies that an anti-IL-4 antibody can block IgE production by >99% without affecting production of other Ig isotypes (37). This observation cannot be explained simply by dose–response considerations and is consistent with the possibility that IL-4, while critical for the stimulation of IgE secretion, may have an auxiliary or redundant role of relatively little importance *in vivo* in generating other humoral immune responses.

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APPENDIX II

Kinetics of Interleukin-4 Induction and Interferon-γ Inhibition of IgE Secretion by Epstein–Barr Virus-Infected Human Peripheral Blood B Cells[1]

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Interleukin-4 (IL-4) acts directly on purified human peripheral blood B cells cultured in the presence of Epstein–Barr virus (EBV) to induce IgE secretion and to enhance the secretion of IgG and IgM. Interferon-γ (IFN-γ) inhibits IgE secretion in this system, without affecting the secretion of the other Ig isotypes. To identify the time period during which EBV-infected B cells can be induced by IL-4 to secrete IgE, we have studied the effects of delayed addition of IL-4, or the termination of IL-4 stimulation by wash out or by neutralization with anti-IL-4 antibodies, on the induction of an IgE response. To induce a maximal IgE response, IL-4 had to be added to cultures of B cells plus EBV no later than 2 days after the initiation of culture, and had to remain present through the tenth day of culture. These two time points correspond to the initiation of detectable DNA synthesis (Days 3 to 4) and the earliest detectable Ig secretion (Days 10 to 12) by EBV-stimulated B cells. No IgE response was induced if the period during which EBV-stimulated B cells were cultured with IL-4 was less than 4 days, or if IL-4 were added later than the tenth day of culture, regardless of how long the culture was continued beyond that time. In contrast, IL-4 considerably enhanced IgG and IgM secretion and B cell CD23 expression, even if it was added after the tenth day of culture. IFN-γ strongly inhibited the IgE response of B cells cultured with IL-4 plus EBV if added within 6 days of the initiation of culture, but had little effect on the generation of IgM or IgG responses made by these cells, regardless of the time of addition. Neither IL-4 nor IFN-γ affected ongoing IgE secretion by an established, IgE-secreting, EBV-transformed cell line. These observations suggest that: (i) IL-4 first becomes able to induce EBV-activated B cells to secrete IgE as these cells begin to synthesize DNA, must stimulate B cells for at least 4 days to induce IgE secretion, and loses its ability to induce IgE secretion as these cells differentiate into Ig-secreting cells; (ii) the ability of IFN-γ to suppress an IgE response is limited to this same time period; and (iii) IL-4 enhancement of CD23 expression and IgM and IgG secretion are independent of IL-4 induction of an IgE response. © 1991 Academic Press, Inc.

INTRODUCTION

The prominent role of IgE in the pathogenesis of atopic disorders (1–3) and its likely role in protective immunity against some helminthic parasites (4–7) have en-

couraged investigation of the regulation of production of this immunoglobulin isotype. Much recent attention has been paid to the roles played by two cytokines, interleukin-4 (IL-4)[2] and interferon-γ (IFN-γ), in this regulatory process. IL-4 has been shown to have pleiotropic effects on the immune system, which include enhancement of B cell class II MHC (8), CD23 (9–12), and CD40 (13) expression, coinduction, with anti-Ig antibodies, of B cell proliferation (14), maintenance of proliferation by some activated T cells (15), and costimulation of IgG1 secretion in mice (16, 17) and IgE secretion in both mice and humans (18–25). The induction of IgE secretion involves a direct effect of IL-4 on the B lymphocyte, since highly purified mouse B cells can be induced to secrete IgE if cultured with IL-4 plus LPS (18), and highly purified human B cells can be induced to secrete IgE if cultured with IL-4 and infected with EBV (23). While IL-4 also enhances IgM and IgG secretion by EBV-infected human B cells, these effects probably occur through a different mechanism than does the stimulation of IgE secretion, which is observed later than the secretion of IgM and IgG and which requires stimulation with higher IL-4 concentrations (23). Higher IL-4 concentrations, and more prolonged stimulation with IL-4, are also required to induce IgE secretion, as opposed to IgG1 secretion, in the murine LPS system (26, 27). In both the murine and human systems, the stimulatory effect of IL-4 on the IgE response can be inhibited by IFN-γ (28–30), which also inhibits many other, but not all, IL-4 effects (31–35).

The present study was undertaken to define the time period during which IL-4 can induce EBV-infected human B cells to secrete IgE and to determine whether the inhibitory effect of IFN-γ on the induction of IgE secretion by EBV-infected cells is limited to the same time period. Possible relationships between the period that IL-4 and IFN-γ influence IgE production and the initiation of DNA synthesis and immunoglobulin production were also investigated. Furthermore, it was of interest to determine whether either IL-4 or IFN-γ can influence IgE secretion by established, IgE-secreting, EBV-transformed B cell lines. Our observations indicate: (i) that IL-4 can only stimulate the development of IgE secreting cells during the period of time following initiation of EBV-induced B cell proliferation and preceding initiation of Ig secretion by these cells; (ii) that IL-4 must be present for a minimum of 4 days during this time period to stimulate IgE production; (iii) that the stimulatory effect of IL-4 on CD23 expression and on IgM and IgG secretion by EBV-infected cells is not limited to this time period; (iv) that IFN-γ has an inhibitory effect on IgE secretion by EBV-infected B cells during the same period that IL-4 has its stimulatory effect; and (v) that neither IL-4 nor IFN-γ influence IgE secretion by an established, IgE-secreting, EBV-transformed B cell line.

MATERIALS AND METHODS

B cell purification. Peripheral blood mononuclear cells were obtained by leukapheresis of healthy normal donors and depleted of polymorphonuclear leukocytes, erythrocytes, macrophages, NK cells, and T cells as previously described (23). In the resulting cell preparation 93–96% of cells bore the B cell-specific surface marker CD20 (B1) (Coulter, Hialeah, FL), while cells that were stained by fluorescein-labeled antibodies specific for T cell, macrophage, or NK-specific markers (Leu 4, Leu M3, Leu 7, and Leu 11, Becton Dickinson, Mt. View, CA) were not detectable (less than 1%).

The al   :e of cells that were stained by these fluores   -labeled antibodies cannot be accounted for by blocking of antigenic determinants by unlabeled mouse mAbs, since no cells were stained detectably by a fluorescein-labeled anti-mouse Ig antibody

Analysis of Ig secretion. IgM. IgG, and IgE levels in supernatants of cultured cells were analyzed by ELISA in 96-well flat-bottomed polystyrene microtiter plates (Dynatech. Alexandria, VA) as previously described (23).

Cytokines. Recombinant human IL-4 was prepared and purified as previously described (23). A concentration of 250 pg/ml of purified IL-4 had one unit of activity, defined as that concentration required to stimulate half-maximal expression of membrane CD23 by the human Burkitt lymphoma Jiyoye cell line. IFN-γ was a gift of the Genetics Institute (Cambridge, MA).

Antibodies. A mouse mAb specific for human CD23. mAb 135 (36), was the generous gift of Dr. G. Delespesse. (Montreal, Canada). A FITC-labeled goat antibody specific for mouse Ig was purchased from Boehringer-Mannheim (Indianapolis. IN). A rabbit antiserum specific for human IL-4 was prepared as described (37). The preparation of a neutralizing mouse mAb specific for human IL-4 will be described in a separate publication (C. Heusser *et al.*, manuscript in preparation).

Culture system. Purified B lymphocytes were infected with EBV by incubation of 10^7 cells at 37°C for 2 hr in 1 ml of culture medium with 3×10^7 EBV infectious units, derived from culture supernatants of the EBV-producing B95.8 marmoset cell line. Cells were then diluted 1:100 in culture medium, after which 10^5 purified B lymphocytes were cultured in 48-well culture plates (Costar. Cambridge, MA) in 1 ml of Hybricare Medium (American Type Culture Collection. Rockville. MD) supplemented with 10% fetal bovine serum (GIBCO, Gaithersberg, MD), 2 mM L-glutamine, 0.5 mg/ml gentamicin, and 2×10^{-5} M 2-mercaptoethanol plus varying concentrations of recombinant cytokines at 37°C in an atmosphere that contained 5% CO_2. Every 7 days 50% of the culture medium was replaced with an equal volume; fresh cytokine, at the initial concentration, was added only with the first replacement of medium.

Flow microfluorimetry. To quantitate CD23 expression by cultured, EBV-transformed B cells, these cells were incubated initially on ice with 1 μg of anti-CD23 mAb or an isotype-matched control mAb, washed, sandwich-stained with FITC-goat anti-mouse Ig antibody, washed again, and analyzed for surface fluorescence with a Coulter Epics 753 flow cytometer, equipped with a three decade logarithmic amplifier.

RESULTS

Effect of delayed addition of IL-4 on the Ig secretory responses made by EBV-infected B lymphocytes. To determine the time period after EBV infection that the presence of IL-4 is first required for the induction of an IgE response. IL-4 was added to purified B cells that were cultured with EBV at the initiation of culture or 2 to 12 days after the initiation of culture, and IgE, IgM, and IgG levels in culture supernatants were determined 22 days after the initiation of culture. In six separate experiments, no IgE was detected (<0.15 ng/ml) in supernatants of cells cultured without IL-4. Addition of IL-4 to cultures 2 days after their initiation generated an IgE response similar to that produced when IL-4 was added at the start of cultures; however, IgE production was decreased by 40% if IL-4 were added 4 days after the start of culture, and decreased further with later IL-4 addition (Fig. 1). In five of these experiments no IgE was produced if IL-4 were added 10 or 12 days after the initiation of culture (Fig. 1). This lack of an IgE response when IL-4 was added 10 or more days after EBV
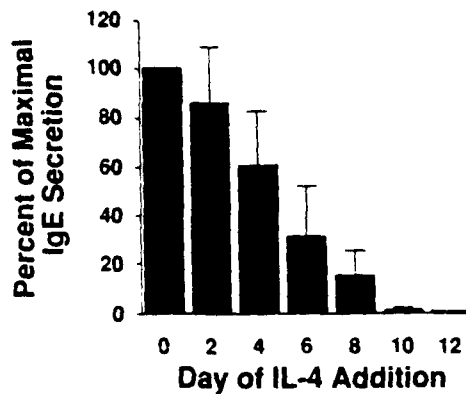
FIG. 1. Effect of delayed addition of IL-4 on the IgE response made by EBV-infected B lymphocytes. Purified human peripheral blood B lymphocytes were infected with EBV and were cultured at 10^5 cells/well in 1 ml of medium. Fifty units/ml of recombinant human IL-4 was added on the indicated day of culture. Day 22 culture supernatants were analyzed for IgE concentration by ELISA. Cultures were performed in triplicate in each experiment. Means and standard deviations of results obtained in six separate experiments are shown. Supernatant IgE concentrations, observed when IL-4 was added at the initiation of culture, ranged from 5.5 to 40.2 ng/ml (mean $\times/\div$ SEM = 13.2 $\times/\div$ 1.51). No detectable IgE (<0.15 ng/ml) was ever found when cells were cultured without IL-4.

infection does not simply reflect an inadequate period of time for IL-4 to have its effects, since these cultures still failed to secrete detectable IgE when the culture period was extended to 32 days (data not shown). In contrast to this requirement for IL-4 relatively early after EBV infection for the generation of a maximal IgE response, addition of IL-4 to a culture of EBV-infected B cells 10 days after initiation still enhanced IgG production 5-fold and IgM production 10-fold (Fig. 2).

Effect of washout or neutralization of IL-4 on the generation of an IgE response by EBV-infected B lymphocytes. To determine the duration of stimulation with IL-4 that
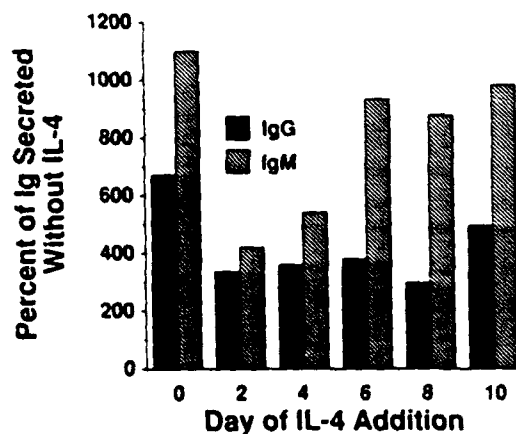

FIG. 2. Effect of late addition of IL-4 on enhancement of IgM and IgG secretion. In a single experiment, purified B lymphocytes were infected with EBV and cultured with or without 50 U/ml of IL-4 as in Fig. 1. Recombinant human IL-4 was added to some cultures at the time points indicated. IgM and IgG levels in culture supernatants after 22 days of culture were determined by ELISA. Means of results from triplicate cultures are shown.

is required to induce EBV-infected B cells to secrete IgE, cultures in which peripheral blood B cells were initially stimulated with both EBV and IL-4 were established. Two to ten days later, IL-4 stimulation was eliminated by either washing the cells and reculturing them in the absence of IL-4 in three experiments (Fig. 3) or by adding a polyclonal rabbit anti-human IL-4 antibody (four experiments) or a monoclonal mouse anti-human IL-4 antibody (two experiments) to the cultures (Fig. 4). Elimination of IL-4 stimulation by either method after 2, 4, or 6 days of culture completely blocked the IgE response. Significant partial inhibition of that response was still observed when IL-4 stimulation was eliminated 10 days after the initiation of culture, while neutralization of IL-4 12 days after the start of culture was no longer inhibitory. Combined with the results of our earlier experiments, these observations localize the stimulatory effect of IL-4 on IgE production by EBV-infected human B cells to a period 2 to 12 days after infection.

To determine the minimum period of time that the presence of IL-4 was required to induce EBV-infected B cells to secrete IgE, IL-4 was added to B cells cultured with EBV at the initiation of culture or 2 to 6 days later, and anti-IL-4 mAb was later added to cultures to neutralize IL-4 activity. In two separate experiments, the generation of an IgE response required that IL-4 be present for 8 days if added at the start of culture, for 6 days if added 2 or 4 days after the start of culture, and for 4 days if added 6 days after the start of culture (Fig. 5). In all cases, further delay in the neutralization of IL-4 resulted in larger IgE responses.

Effect of time of addition of IFN-γ on Ig secretion by B cells cultured with EBV + IL-4. To determine if the ability of IFN-γ to inhibit the IgE response of B cells cultured with EBV and IL-4 correlated temporally with the ability of IL-4 to induce this response, IFN-γ was added to B cells cultured with EBV plus IL-4 at the initiation
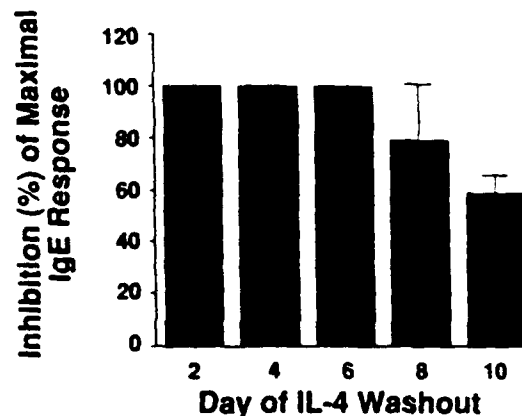

FIG. 3. Determination of the duration of the IL-4 requirement for generation of an IgE response by EBV-infected B cells by IL-4 washout. Purified human peripheral blood B cells were infected with EBV and were cultured with IL-4 as in Fig. 1. At the time points indicated, cells were aspirated from culture wells, washed three times with 5-ml aliquots of cold PBS supplemented with 1% fetal bovine serum, resuspended in culture medium, and recultured without adjusting for cell number. Culture supernatants were harvested 22 days after the start of the initial culture and assayed by ELISA for IgE concentration. Means and standard deviations of the results obtained in three experiments are shown as percentage inhibition by washout of the IgE responses obtained when cells were cultured with IL-4 for the entire 22-day period. Supernatant IgE concentrations observed when IL-4 was present for the entire period of culture ranged from 5.5 to 40.2 ng/ml (mean ×/÷ SEM = 13.5 ×/÷ 1.8).
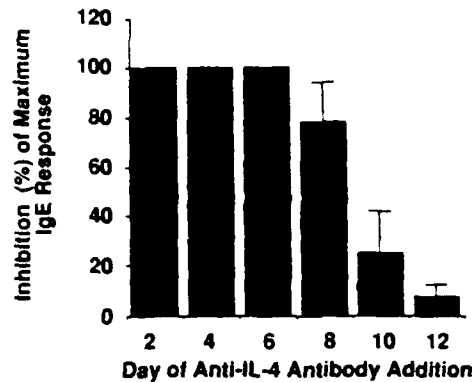
FIG. 4. Determination with anti-IL-4 antibodies of the duration of the IL-4 requirement for generation of an IgE response by EBV-infected B cells. Purified human peripheral blood B cells were infected with EBV and were cultured with IL-4 as in Fig. 1. At the time points indicated, rabbit anti-human IL-4 antiserum (a 1:5000 dilution) or a purified, mouse anti-human IL-4 mAb (5 μg/ml) was added to cultures. Culture supernatants were harvested on Day 22 and IgE concentrations were determined by ELISA. Means and standard deviations of the results obtained in six experiments (four performed with rabbit anti-human IL-4, two with mouse anti-human IL-4) are shown as percentage inhibition of the response obtained when no anti-IL-4 antibody was added to cultures. Supernatant IgE concentrations observed in different experiments when no anti-IL-4 antibody was added ranged from 2.5 to 40.2 ng/ml (mean ×/÷ SEM = 14.9 ×/÷ 1.8). Addition of control rabbit or mouse antibodies to cultures had no effect on IgE production (data not shown).

of culture or 2 to 10 days later. IgM, IgG, and IgE levels were determined after 22 days of culture. In three separate experiments, IFN-γ added as late as the sixth day of culture inhibited IgE production by an average of 60%, while addition on the eighth day of culture inhibited IgE production by an average of 40%, and addition on Day 10 caused less than 20% inhibition (Fig. 6). The ability of IFN-γ to inhibit IgM and IgG secretion in this system, determined in a single experiment, was much more limited; little or no inhibition was noted when this cytokine was added 2 or more days
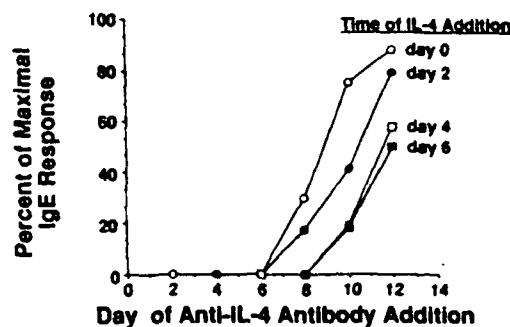

FIG. 5. IL-4 must be present for at least 4 days to induce EBV-infected B cells to secrete IgE. Purified human peripheral blood B cells were infected with EBV and were cultured as in Fig. 1. IL-4 (50 U/ml) and mouse anti-human IL-4 mAb (5 μg/ml) were added at the time points shown. Culture supernatant IgE concentrations were determined by ELISA after 22 days of culture. Data are shown as means of three replicate cultures in each of two separate experiments for each time point, compared to the supernatant IgE when IL-4 was present and anti-IL-4 mAb absent for the entire 22-day culture period (4.2 and 31 ng/ml in the two studies).
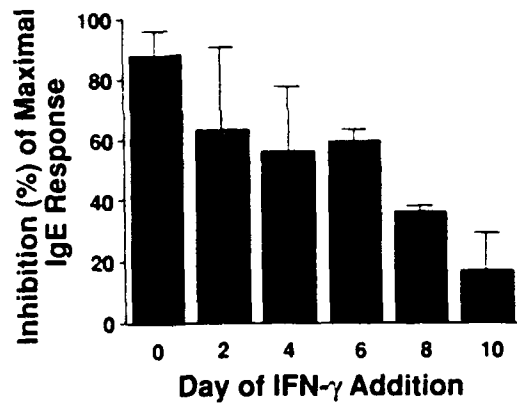
FIG. 6. Effect of time of addition of IFN-γ on IgE secretion by B cells cultured with EBV plus IL-4. Purified human peripheral blood B cells were infected with EBV and were cultured with 50 U/ml of IL-4 as in Fig. 1. IFN-γ (20 U/ml) was added at the time points indicated, and culture supernatant IgE concentrations were determined by ELISA after 22 days of culture. Data are expressed as the percentage suppression of the IgE response induced by EBV plus IL-4 in the absence of IFN-γ. Means and standard deviations of results from three separate experiments, performed with the studies illustrated in Fig. 3 are shown. Cultures to which no IFN-γ was added ranged from 5.5 to 40.2 ng/ml (mean ×/÷ SEM = 13.5 ×/÷ 1.8).

after the initiation of culture (Fig. 7). These data confirm our previous observation that the suppressive effect of IFN-γ in this system is specific for IgE (23), and indicate that the time period during which IFN-γ is able to suppress IgE production is identical to the period during which IL-4 can stimulate IgE production.

IgE secretion by EBV-infected B cells is influenced by IL-4 and IFN-γ between the onset of EBV-induced B cell proliferation and Ig secretion. Our above observations indicate that the specific effects of IL-4 and IFN-γ on IgE production by EBV-infected B lymphocytes first become demonstrable approximately 2 days after the addition of
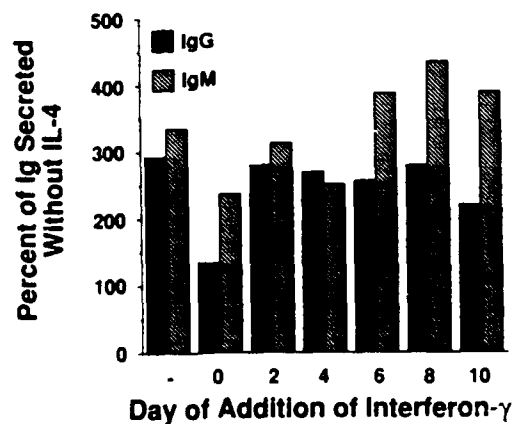

FIG. 7. Effect of time of IFN-γ addition on IgM and IgG secretion by B cells cultured with EBV plus IL-4. Serum IgM and IgG levels in Day 22 culture supernatants from one of the experiments shown in Fig. 6 were determined by ELISA. IFN-γ was added to cultures at the times indicated or was omitted from cultures (–). Data are expressed as means of three replicate cultures for each time point, compared to the IgG and IgM responses made by EBV-infected cells cultured with neither IL-4 nor IFN-γ.

EBV to B cells and are no longer demonstrable if these cytokines are added more than 10 to 12 days after the initiation of infection. To determine whether the timing of this responsiveness might correlate with important events in B cell proliferation and differentiation that follow infection of B cells with EBV, we determined the timing of DNA synthesis ([³H]thymidine incorporation) and IgM secretion by purified B cells cultured with EBV plus IL-4. DNA synthesis was first detectable 3 to 4 days after the initiation of culture, while IgM was first detected in culture supernatants after 10 to 12 days (Fig. 8). Thus, the IL-4 stimulatory effect on IgE production by EBV-infected B cells first becomes evident as these cells begin to synthesize DNA. and both the IL-4 stimulatory effect and the IFN-γ inhibitory effect on IgE production are lost as these cells begin to secrete Ig.

EBV-transformed, IgE-secreting B cells respond to IL-4 with increased CD23 expression, but neither IL-4 nor IFN-γ modulate IgE secretion by these cells. To determine the abilities of IL-4 and IFN-γ to affect IgE production by an established, IgE-secreting, EBV-transformed B cell line, B cells were initially cultured for 5 weeks with EBV plus IL-4, then washed and recultured for 1 week in the absence of IL-4. Individual cells in the resulting EBV-transformed cell population were secreting IgM, IgG, or IgE (data not shown). Cells from this population were recultured a second time with no added cytokine, IL-4, IFN-γ, or both cytokines, and culture supernatants were harvested and assayed for IgE every 5 days for the next 2 weeks (Fig. 9). No effects of these cytokines on IgE production in this system were observed. Thus, IFN-γ loses its ability to inhibit IgE secretion in this system once EBV-transformed B cells become established as secretors of IgE, and IL-4 is unable to stimulate IgE secretion by EBV-transformed B cells that have become committed to the secretion of a different isotype, or to enhance IgE secretion by IgE-committed, EBV-transformed B cells. To determine whether the inability of IL-4 to enhance IgE secretion by established, EBV-transformed B cells might be due to either the loss of IL-4 receptor by these cells or the inability of this receptor to signal transformed cells, we investigated the ability of IL-4 to exert another of its known effects on these cells: enhancement of CD23 expres-
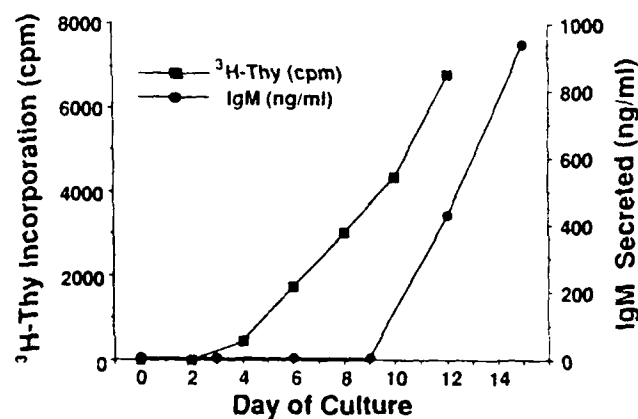

FIG. 8. Kinetics of DNA synthesis and IgM secretion by purified B lymphocytes cultured with EBV. Purified human peripheral blood B cells were infected with EBV and cultured with 50 U/ml of IL-4 as described for Fig. 1. Cells were pulsed for 6 hr with 1 μCi of [³H]thymidine, at the time points indicated, to detect DNA synthesis. Culture supernatants harvested at the time points indicated were assayed by ELISA for IgM concentration. All values shown are means obtained from three replicate cultures.
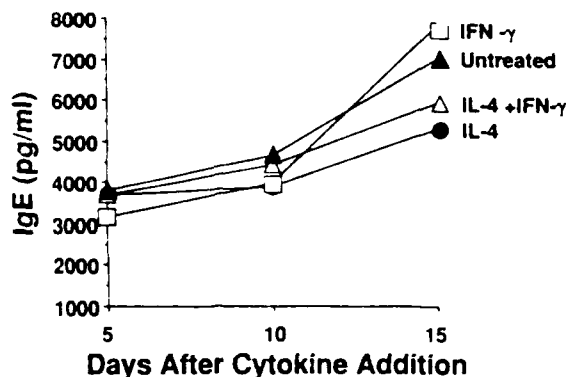
FIG. 9. IL-4 and IFN-γ do not affect IgE secretion by established. IgE secreting. EBV-transformed B cells. Purified human peripheral blood B cells were infected with EBV and cultured with IL-4 (50 U/ml) for 35 days. then washed and cultured for an additional week without added cytokines. Cells were then washed again and cultured at 10^5/ml with no additional cytokines. IL-4 (50 U/ml). IFN-γ (20 U/ml). or both IL-4 and IFN-γ. Culture supernatants were harvested 5. 10. and 15 days after the initiation of the final culture, and were assayed for IgE concentration by ELISA. The means of values obtained from triplicate cultures are shown.

sion (9-12). Although B cells cultured for 16 days with EBV in the absence of IL-4 express considerably more CD23 than do unstimulated human peripheral blood B cells (38). culture of these EBV-transformed cells for an additional 2 days with IL-4 stimulated an additional. approximately fourfold. increase in CD23 expression (Fig. 10). Thus. the inability of IL-4 to affect IgE secretion by B cells once they have been transformed by EBV is not a result of IL-4 receptor loss or a loss of the ability of this receptor to transmit a signal to the B cell.
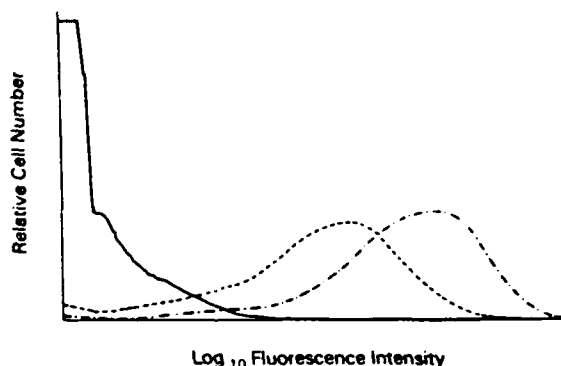

Log $_{10}$ Fluorescence Intensity

FIG. 10. IL-4 induces EBV-transformed B cells to increase their membrane expression of CD23. EBV-infected human peripheral blood B lymphocytes were cultured without IL-4 for 16 days. after which 50 U/ml of IL-4 was added to one of two cultures. After an additional 2-day culture period cells were stained with either a mouse anti-human CD23 mAb or an isotype-matched control mAb. followed by FITC-labeled goat anti-mouse Ig antibody. and analyzed for surface fluorescence with a flow microfluorimeter equipped with a three decade log amplifier. The fluorescence profile of cells stained with the control antibody is shown as a solid line: that of IL-4-treated cells stained for CD23 is shown as a dashed line: and that of cells cultured without IL-4 and stained for CD23 is shown as a dotted line. IL-4 treatment did not affect the fluorescence profile of cells stained with the control antibody (not shown).

DISCUSSION

The critical role of IgE in the pathogenesis of atopic disorders has focused attention on mechanisms that regulate the production of this immunoglobulin isotype. Several studies in the mouse and in human have established that the T cell-produced cytokine IL-4 is an important inducer of IgE responses (18–25). By contrast IFN-γ has been shown to inhibit IgE production. However, little is known about the mechanism of action of both cytokines. In this study we try to delineate the timing requirement and the stages during B cell activation that IL-4 and IFN-γ can affect IgE production. We utilize a system in which purified B lymphocytes are activated by infection with EBV and induced by IL-4 to secrete IgE.

We show that IL-4 has to be present for a relatively extended period of time, at least 4 days, to induce any IgE production, and that maximum production is seen when IL-4 is present for 8–10 days, starting the first 2 days of culture. Neutralization or washout of IL-4, added at the beginning of culture 6 days later, completely suppressed IgE production. Addition of IL-4 after the tenth day of culture also failed to induce detectable IgE production. In these same experiments DNA synthesis, as detected by [^{3}H]thymidine incorporation, starts approximately 3 days after the initiation of culture, and IgM is first detected in measurable quantity 7 days later. Taken together these observations suggest that IL-4 can act as an IgE inducing factor on activated B cells that have already started to synthesize DNA but have not yet terminally differentiated into Ig producing plasma cells. After this stage the loss of effect of IL-4 on the stimulation of IgE secretion is not a result of a total loss of IL-4 responsiveness by these cells. IL-4 can still enhance IgM and IgG secretion by EBV-infected cells, an effect that is probably due to enhanced cell proliferation or survival (23), after it loses the ability to stimulate an IgE response. In addition, most B cells, after culture with EBV for 16 days, still increase their expression of CD23 when IL-4 is subsequently added to the culture medium, even though no IgE secretion is induced. This observation does not exclude the possibility that precursors of IgE-secreting cells are distinct from precursors of cells that secrete other Ig isotypes and that precursors of IgE secreting cells, unlike precursors of IgG and IgM-secreting cells, lose all responsiveness to IL-4 after EBV-induced transformation. Since there is no evidence, however, that precursors of IgE-secreting cells represent a distinct cell population, we favor the view that the loss of effect of IL-4 on IgE secretion as EBV-infected cells start to secrete Ig results from a loss in the ability of IL-4 to induce the molecular events that lead to IgE production.

The 4-day period of exposure to IL-4 that is required for the induction of any IgE secretion by human B cells cultured with EBV and the 8- to 10-day period of exposure that is required for maximum IgE production, as opposed to the less than 24-hr exposure to IL-4 that is sufficient to induce events such as increased CD23 or CD40 expression (13), are in agreement with and extend previous findings in mice. These studies have demonstrated that IL-4 needs to be added to cultures no later than 1 day after initial stimulation with LPS, and must remain in the culture for a period of 3 days to induce IgE production (26, 27). However, IL-4 stimulates increased Ia expression by mouse B cells in less than 24 hr (8). The observations on both mouse and human support the view that IL-4 may be required to stimulate more than one molecular event during B cell differentiation to induce IgE commitment. Two such molecular events might be an increase in the accessibility of the Cε gene, which is reflected by the synthesis of a sterile Cε transcript, and which has been shown to be IL-4 inducible

in a mouse syst39–41), and a recombinational event that deletes C_H genes located between VDJ ai. Ce. Recent reports indicate that IL-4 induce V-infected B cells to develop into stable. IgE-secreting cells that have deleted the $C\mu$ and $C\gamma$ loci (42, 43). It has also recently been shown that human PBL stimulated with IL-4 express a 1.75-kb germline $C\epsilon$ mRNA transcript after 4 days in culture and that a 2.35-kb mature mRNA appears later (44). While IL-4 is known to induce expression of the sterile $C\epsilon$ transcript it is not yet known whether the continuous presence of IL-4 is required for deletion of the C_H genes between VDJ and $C\epsilon$ and expression of the mature $C\epsilon$ transcript.

In addition to localizing the time of IL-4 action in the induction of IgE secretion our experiments demonstrate that IFN-γ inhibits IgE production when added early to cultures of EBV plus IL-4 stimulated B cells, and is ineffective when added after Day 10, even though IgE secretion has not yet begun. These observations strongly suggest that IFN-γ inhibits commitment of B cells to IgE production rather than production of IgE by already commited cells. Furthermore, since IgE production by EBV-transformed cells continues for a long time, it was possible to determine whether ongoing IgE production could be modulated by IFN-γ and/or IL-4. Our results show that neither cytokine substantially influences ongoing IgE production by EBV-transformed cells. No modulation of ongoing IgE secretion by IL-4 and IFN-γ has also been observed in some, but not all, studies using nontransformed PBL from hyper-IgE syndrome and parasite-infected patients ((45), and J. de Vries and C. King, personal communications).

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